

Identification and Expression of a Murine Cytomegalovirus Early Gene Coding for an Fc Receptor

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Several herpesviruses, including cytomegalovirus, induce receptors for the Fc domain of murine immunoglobulin G (IgG) molecules. Viral genes coding for these receptors have been characterized only for alphaherpesviruses. In this report, we describe a new approach that led to the identification of an Fc receptor (FcR) of murine cytomegalovirus (MCMV). The Fc fragment of IgG precipitated glycoproteins (gp) of 86 to 88 and 105 kDa from MCMV-infected cells. Deglycosylation by endoglycosidase F resulted in a protein with a molecular mass of 64 kDa. Injection of complete MCMV DNA or of DNA fragments, and the subsequent testing of cytoplasmic binding of IgG by immunofluorescence microscopy, was used to search for the coding region in the MCMV genome. The gene was located in the *Hind*III J fragment, map units 0.838 to 0.846, where an open reading frame of 1,707 nucleotides predicts a gp of 569 amino acids with a calculated molecular mass of 65 kDa. The sequence of this gp is related to those of the gE proteins of herpes simplex virus type 1 and varicella-zoster virus. The defined length of the mRNA, 1,838 nucleotides, was in agreement with that of a 1.9-kb RNA expressed throughout the replication cycle, starting at the early stages of infection. Expression of the gene *fcrl* by recombinant vaccinia virus resulted in the synthesis of gp86/88 and gp105, each with FcR properties, and the correct identification of the gene encoding the FcR was confirmed by the DNA injection method.

Fc receptors (FcR) are a group of cellular surface molecules that specifically recognize and bind homologous immunoglobulin via the Fc part and which mediate various biological functions. For each immunoglobulin heavy chain, there are corresponding FcR expressed on specific cells of all the major hematopoietic lineages. FcR for immunoglobulin G (IgG) form a heterogeneous group and link specific humoral and cellular immune responses and thus confer antigen specificity to phagocytic effector cells that lack receptors specific for antigen (51). The functions triggered by FcR include phagocytosis, endocytosis, antibody-dependent cellular cytotoxicity, release of inflammatory mediators, and modulation of antigen presentation. Three types of murine and human FcR can be distinguished. All three receptors belong to the immunoglobulin supergene family, are associated with homo- and heterodimers, and also exist as soluble receptors (52).

FcR are also expressed by some bacteria and have been typed according to their functional reactivities with different species and subclasses of IgG (6). Remarkably, induction of FcR is also a property of certain coronaviruses (38) and of herpesviruses, including herpes simplex virus type 1 (HSV-1) and HSV-2 (1, 3, 54), varicella-zoster virus (VZV) (37), cytomegalovirus (CMV) (21, 27, 40, 55), and Epstein-Barr virus (57). Baucke and Spear (3) identified Fc-binding glycoprotein gE of HSV-1. In order to achieve full FcR activity, gE forms a complex with a second glycoprotein, gI (4, 23-25). The FcR genes of the herpesviruses are not members of the immunoglobulin supergene family.

The biological role of FcR expressed by herpesviruses is unclear. The present concept, which is supported by *in vitro* data, is that the HSV-1 FcR is active in bipolar bridging of

specific IgG (18), which reduces the efficacy of virus neutralization (12) and antibody-dependent cellular cytotoxicity (15). Induction of an FcR by a herpesvirus may also have adverse effects, since FcR induction by CMV has been reported to confer human immunodeficiency virus susceptibility to cells (34).

We recently observed that in B-cell-deficient mice, the primary infection by murine CMV (MCMV) takes the same course in the presence and absence of antibody (26). One explanation could be that functions specified by the virus, for instance, induction of FcR, could modulate the activity of antibodies. In order to test the potential immunological role of FcR, we therefore set out to identify genes coding for a potential FcR activity of MCMV. This should enable us to construct and test CMV mutants that harbor or lack this function. In this report, we describe the identification and genomic location of a gene coding for FcR, determined by a method involving injection of viral genomic DNA fragments and monitoring of the expression phenotype. The gene was sequenced and the protein was expressed by vaccinia virus.

MATERIALS AND METHODS

Cells, virus, and infection conditions. BALB.SV, a immortalized cell line of BALB/c murine embryonal fibroblasts (MEF) (11), was grown in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) fetal calf serum. MCMV (strain Smith, ATCC VR-194) was propagated on MEF as described previously (44). The cells were infected by the technique of centrifugal enhancement of infectivity.

For selective expression of immediate-early proteins, the cells were infected in the presence of cycloheximide (50 µg/ml), which was replaced with actinomycin D (5 µg/ml) after 3 h of incubation (10). For selective expression of early proteins, the cells were infected in the presence of phosphonoacetic acid (250 mg/ml), an inhibitor of DNA synthesis.

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Preparation of viral DNA. The MCMV DNA was prepared from the infected cells and purified by CsCl gradient centrifugation as described previously (16).

Microinjection. The cells were seeded on Cell locates (Eppendorf, Hamburg, Germany), and individual cells were injected with 50 to 100 ng of genomic or plasmid DNA per μ l. The automatic injection system from Eppendorf was used by following the instructions of the manufacturer. Cells were injected into the nucleus for 0.5 s at a constant pressure of 80 hPa. Under these conditions, approximately 0.05 pl of the sample was injected, corresponding to about 200 copies of plasmid DNA. Per Cell locate, about 150 cells were injected. After injection, the cells were incubated for a further 24 h at 37°C and 5% CO₂. The microinjected cells were fixed for 20 min in 3% (wt/vol) paraformaldehyde, and free groups were quenched for 10 min in 50 mM NH₄Cl. After permeabilization with 0.1% Triton X-100 for 5 min and a washing in phosphate-buffered saline, the cells were incubated with murine monoclonal IgG for 30 min at room temperature (35). As a secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) was used.

Metabolic labeling and protein analysis. Monolayers of cells grown in 6-cm-diameter petri dishes were infected with MCMV (multiplicity of infection of 20) or with recombinant vaccinia viruses (multiplicity of infection of 2). The cells were pulse-labeled with 250 μ Ci of [³⁵S]methionine (Amersham, Braunschweig, Germany) per ml in methionine-free Dulbecco's modified Eagle medium supplemented with 5% dialyzed fetal calf serum and were chased in medium containing unlabeled methionine. Labeled cells were lysed on ice in a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Cytoplasmic extracts were incubated with murine IgG for 1 h at 4°C, and immune complexes were precipitated with protein A-coupled Sepharose (Pharmacia, Uppsala, Sweden). Bound complexes were eluted by heating at 96°C for 5 min in a reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and analyzed by 8% PAGE. In some experiments, Sepharose-coupled immunoglobulin fragments and different immunoglobulin allotypes were used. The coupling of cyanogen bromide-activated Sepharose (Pharmacia) was done according to the instructions of the manufacturer. Affinity-purified mouse IgG, mouse IgG Fc fragments, and mouse IgG F(ab)₂ fragments were obtained from Dianova. *N*-Glycosidase F (PGNase F; Boehringer, Mannheim, Germany) digestion was performed as described previously (2). The precipitated proteins were denatured and digested with PGNase F (1 U/ml) overnight.

DNA sequence analysis. The nucleic acid sequence was determined by a modified version of the dideoxy sequencing method (50). Both strands were sequenced with subclones and specific primers. The Genetics Computer Group software package, version 7.2, was used for the analysis of the nucleic acid sequences and for the deduction of the amino acid sequence.

RNA analysis. The cells were infected with MCMV at a multiplicity of infection of 10. Total cellular RNA was isolated at different times postinfection (p.i.) according to the method of Chirgwin et al. (8). Denatured RNA samples were size fractionated by agarose gel electrophoresis and blotted onto a nylon membrane (GeneScreen; NEN). Filters were prehybridized for 3 h at 68°C in 5 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5 \times Denhardt's solution (1 \times Denhardt's solution is 0.02% [each] bovine serum albumin [BSA], polyvinylpyrrolidone, and Ficoll 400)–1% SDS–10% dextran sulfate–250 μ g of salmon sperm DNA per ml. Hybridization

was carried out overnight, under the same conditions, with radioactively labeled probes (46). A probe identical to the coding strand was generated on the template of the *Bam*HI-*Eco*RI fragment (map units 0.837 to 0.843) with primer 5'-CGCATAAGGTAGGACCAC-3', and a probe identical to the opposite strand was generated on the template of the *Bam*HI-*Bgl*II fragment (map units 0.848 to 0.841) with primer 5'-AGACAGACCATGCCATCG-3'. Full-length cDNA from the human fibroblast cytoplasmic β -actin gene (22), labeled with [α -³²P]dCTP by using a randomly primed DNA labeling system (Boehringer), served as a probe for a cellular gene.

Primer extension. The 5' end of the mRNA was determined by primer extension. RNA (20 μ g) was mixed with the primer end labeled with ³²P (5'-GGTAATTGATGCGCCGTCACG GCCG-3') in a hybridization buffer (2 M NaCl, 50 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.8], 0.05% SDS), incubated for 5 min at 65°C, and then hybridized at 37°C for 5 h. The hybridized RNA was purified by ethanol precipitation and subjected to reverse transcription with avian myeloblastosis virus reverse transcriptase (Boehringer). The same primer was used for the sequencing reaction. The sequencing products and the cDNA were run on a 6% polyacrylamide gel, and the gel was subjected to autoradiography.

Determination of the 3' end of the mRNA. The 3' end of the MCMV FcR mRNA was determined according to the method of Frohman et al. (20). The cDNA was synthesized by reverse transcription with oligonucleotide (dT)17-R1-R0 (5'-AAGGA TCCGTCGACATCGATAATACGACTCACTATAGGGAT TTTTTTTTTTTTTTTT-3'), and the transcribed product was then subjected to PCR amplification with oligonucleotides R0 (5'-AAGGATCCGTCGACATC-3') and B23-14.3-*Hind*III (5'-CCGAAGCTTCTCCATCTCGATCAACGG-3'). The primers contained the restriction sites, which allowed the cloning and sequencing of the amplified product.

Production of recombinant vaccinia viruses. Recombinant vaccinia virus expressing the complete MCMV FcR open reading frame (ORF) was generated as described previously (32). Briefly, the 2,373-bp *Bam*HI-*Bam*HI fragment was cloned into the vaccinia virus expression vector p7.5K, which allows the expression of foreign proteins under the control of the 7.5-kDa early-late promoter. The virus recombinants were produced with the DNA of vaccinia virus strain Copenhagen. Recombinants expressing their respective MCMV FcR ORFs were selected by infecting HU TK⁻ 143 cells.

Nucleotide sequence accession number. The EMBL Data Library accession number for the nucleotide sequence reported in this article is X77798.

RESULTS

Identification of an MCMV early glycoprotein with IgG binding properties. A cytoplasmic FcR activity expressed following infection with human CMV has been reported by Furukawa et al. (21). The expression of MCMV-induced immunoglobulin binding activity in the infected cells was tested accordingly. By indirect immunofluorescence, a strong cytoplasmic staining was observed with purified murine IgG but not with murine IgM, indicating that MCMV encodes a function similar to that encoded by human CMV (Fig. 1). No signal occurred when only the labeled antiserum was used. The fluorescence signal became detectable 5 to 6 h after infection and reached plateau levels at about 10 h p.i. Infection of cells in the presence of phosphonoacetic acid resulted in the same phenotype except when immediate-early genes were expressed because of the subsequent use of cycloheximide and actinomycin.

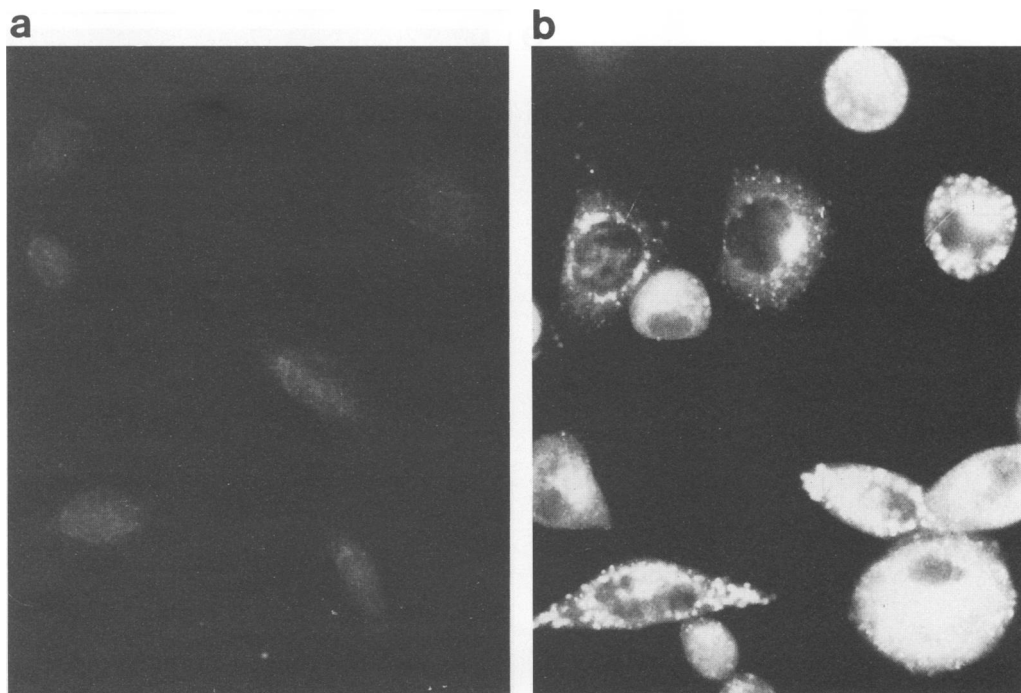


FIG. 1. Immunoglobulin binding activity induced by MCMV infection. Noninfected cells (a) and MCMV-infected cells (b) were stained with mouse IgG and subjected to indirect immunofluorescence.

cin D, indicating that induction of IgG binding is an early-phase function (data not shown).

To identify proteins with immunoglobulin binding properties, the cells were pulse-labeled with [35 S]methionine at 12 h after infection and chased for 10, 20, and 30 min. Precipitation with purified mouse IgG showed proteins with apparent molecular masses of 86 and 88 kDa. Some of this material was processed to a protein of 105 kDa after 20 min of chase (Fig. 2a). Precipitation and subsequent digestion with endoglycosidase F increased the electrophoretic mobilities of the 86- and 88-kDa species and the 105-kDa species to a band of 65 kDa (Fig. 2b), indicating that the immunoglobulin-binding protein is an early glycoprotein.

To test whether the capacity to bind immunoglobulins is restricted to the Fc portion of the immunoglobulin molecule, Sepharose particles were coated with purified Fc fragments, purified F(ab) $_2$ fragments, and complete IgG molecules. Affinity precipitation showed that gp86/88 is precipitated by IgG Fc fragments but not by IgG F(ab) $_2$ fragments (Fig. 2c). Therefore, binding of gp86/88 and gp105 is specific for the Fc region of the IgG molecule, leading to the conclusion that MCMV induces a receptor for the Fc fragment of IgG.

Identification of the FcR gene by microinjection of MCMV DNA fragments. In order to localize the FcR gene within the MCMV genome, we used a DNA microinjection method and monitored the intracellular expression of an IgG binding activity by indirect immunofluorescence. The general strategy of the microinjection approach is outlined in Fig. 3. Twenty-four hours after injection of complete undigested MCMV DNA (Fig. 3a), a strong expression of FcR in the cytoplasm can be seen (Fig. 3e). Perinuclear localization of large cytoplasmic vacuolar structures suggests the accumulation of FcR protein in a distinct cytoplasmic compartment. The detection of this pattern of fluorescence was used in further experiments to locate the genomic position of the gene.

To this end, the MCMV DNA was first cleaved with various restriction enzymes and the DNA fragments were injected (Fig. 3b). This type of analysis was considered to indicate which restriction enzyme would cleave either the gene of the

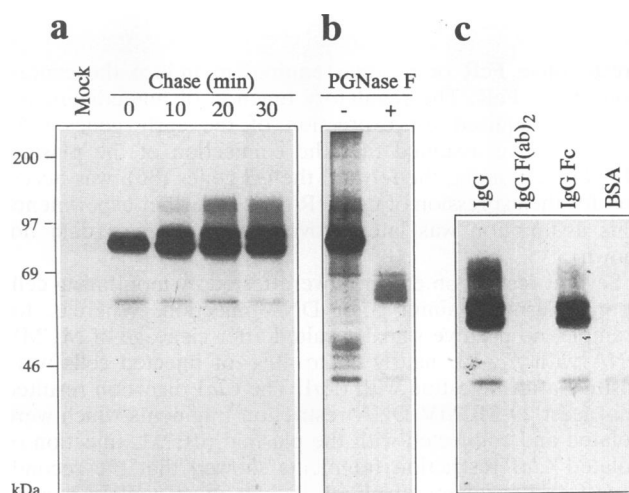


FIG. 2. Precipitation of glycoproteins with specificity for Fc portion of mouse IgG from MCMV-infected cells. (a) Cells were pulse-labeled for 10 min with [35 S]methionine at 12 h p.i. and chased for the indicated period of time. Mock-infected cells were chased for 30 min. The precipitation from cytoplasmic extracts was done with purified mouse IgG and protein A-Sepharose. (b) A sample precipitated with IgG as for panel a was split into two fractions, and the smaller fraction (+), containing about 25% of the material, was digested by endoglycosidase F. (c) The MCMV-infected cells were pulse-labeled for 1 h, and the lysates were precipitated with Sepharose-coupled IgG, IgG F(ab) $_2$, IgG Fc, or BSA.

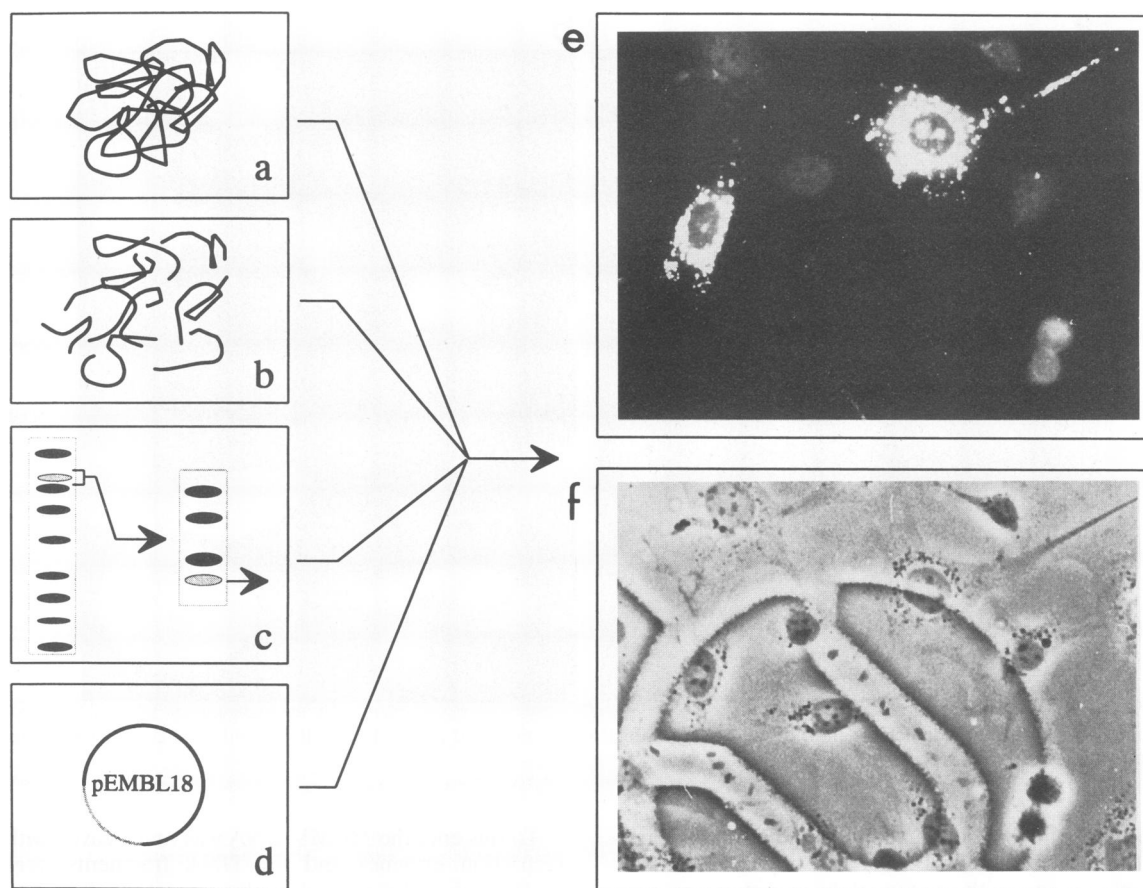


FIG. 3. Strategy for the identification of the MCMV FcR gene. Undigested MCMV DNA (a), restriction enzyme-digested DNA (b), eluted DNA fragments (c), and cloned restriction fragments (d) were injected into the nuclei of the cells. (e) The expression of proteins binding a mouse monoclonal antibody of the IgG2a subtype was determined by immunofluorescence. (f) Injected cells were localized with CellLocate.

presumptive FcR or a gene required to induce the expression of the FcR. The regulatory function of immediate-early genes is required for expression of the early genes (36). Therefore, we assumed that the coinjection of the plasmid pIE111, containing the *ie1* and the *ie3* genes (36), was necessary for the expression of the FcR gene in further experiments. This assumption was later proved to be correct (data not shown).

Several restriction enzymes were tested by monitoring cells by cytoplasmic staining after DNA injection. Whereas, for example, no positive signal resulted after cleavage of MCMV DNA with *EcoRI*, nearly 30 to 40% of injected cells were positive after digestion with *ClaI*. The *ClaI* digestion resulted in at least 29 MCMV DNA restriction fragments which were isolated and coinjected with the plasmid pIE111. Injection of isolated *ClaI* restriction fragments showed that the second-largest (B) fragment of about 12 kbp must contain a gene which either encodes or induces the FcR protein (Fig. 3c). This fragment was cloned into the pACYC177 vector, and subfragments of the cloned fragment were retested. Injection of various restriction subfragments led to the conclusion that the minimal fragment which still induced the expression of the FcR function is a 2.4-kbp fragment produced by digestion with *BamHI* and containing the expected *EcoRI* site (Fig. 3c). This fragment was subcloned into the pEMBL18 vector for nucleotide sequencing (Fig. 3d).

Nucleotide and amino acid sequences of the gene encoding FcR. The 2.4-kbp *BamHI* subfragment of the *ClaI* B fragment was sequenced with specific primers (Fig. 4). The sequence analysis revealed only one ORF (1,707 nucleotides [nt]) with the potential to encode a protein of 569 amino acids and a calculated molecular mass of 64 kDa. This prediction was in good agreement with the result obtained after precipitation of the deglycosylated protein. The first ATG was found 87 nt downstream from the TATA box in a favorable context for initiation of translation (29). The polyadenylation consensus sequence was located 56 nt downstream from the stop codon. Computer-aided analysis with the Genetics Computer Group sequence analysis software package, version 7.2, showed no obvious nucleotide sequence homology to any known sequence or to sequences of genes from members of the herpesvirus family. The gene was designated *fcrl*.

The amino acid sequence of the MCMV FcR was deduced from the nucleotide sequence of the *fcrl* ORF. The hydrophobic and hydrophilic domains of the protein were analyzed. A hydrophobic region at the N terminus represents a putative signal peptide sequence of 17 residues, according to the algorithms of von Heijne (53). A second hydrophobic region of 18 amino acids probably representing the transmembrane region, which is followed by a 17-amino-acid cytoplasmic tail, was identified at the C terminus (positions 535 to 552). The

1 ggatccccgaggaagccttcgaatccctgtcgggacagacaggcgatcccgatacccg
 60 gggctctgcgacgctcctgtcgtgctccgacgagcgttctatcgtcgttacgtccg
 120 ccatggacatgttcgacggcggaagagagagcgagcgagattatgttcacagg
 180 ccgagagggtcggtcgtccgaagacgggaacccattgaaactgccacgtctggtcc
 240 ggcgactcaggcggtatcgacgagcaacaacatggaacggctcggatcgtagtcca
 300 gggcgctgtgtcagcgctcggggggggcgcgacacacatcagtcataaaagccg
 360 tgccgcatcacagaaactcTGTCACAGAGCGGAGCTCAAGTCGCCATCTCTCGGTC
 420 GGCAGAGCGGAGCGATGGCGCTTCGACGCTGATCGACTCTGCCTCCGCGGCGTG
 1 M A P S T L I A L C L L A A V
 480 ACGGCGCATCAATTACCTGCGTGCCAGTGGCGCTTGCCACAGAGACCTCCGTCCTC
 16 T A H Q L P A C Q W R L P T E T S V P F
 540 AATATCTCTCGGACCTGGTGAACGGCTCCACGGTCAAGGTCTGGCGAGACCTCTGCGGC
 36 N I S C D L V N G S T V K V W R D L C G
 600 GAAGCAATGCTACCGAGGATGGATCGGGATGGCGCGCGGAGACAAATACATTTTC
 56 E G N A T E G W I G M A R R G D K L H F
 660 TCCGCGAGGTGAATCTCGACCGCGACATCGGGTCTACTACGTCAACGTGACCGGAG
 76 S A R W N Y S T A D I G V Y Y V N V T E
 720 CGTAACCTGACGACTCCCTGACGCACTGATCACCCACCGATGACGATCTGGGCAATG
 96 R N L T T P L T Q L I T P P M T I W G M
 780 AGGAGGGCGGAGAGCAGAGACTTTGTCCTTGCACCGTGACGGGATCTTCTCGAAG
 116 R Q G G R S R D F V L C T F V T G I F S K
 840 GGTGAATTCACACTCGAGGCGAAGCGCGGACGGTCTCTCTGTCACTTCACAAAACCC
 136 G E F T L E A N G A T V L S V N F T K P
 900 GGACGATATCGAGTGAAGGCGCGCTCTCTAGACCTCTCACATCGCAACGGTACC
 156 G R Y A V K K A R V S L D L S H R N G T
 960 ATGCGATTCTCGTCTCGATGAGGGGGCCCATGACGACGTGAATATCATGTCATC
 176 M R F L V S I R G G P M T H V K Y Q C I
 1020 ATGACTCTCTCTCGTGGTCTCTTTGGCGTACGACCGCTGTGATGTTGAATACAGA
 196 M T P L S R P F A V S K P L M L E Y R
 1080 CAGACCATGCCATCGGGAGTGAAGGATACCAAGGACTCCACGGGACTGAAGTCGCC
 216 Q T M P S G V K R Y T K D S T G T E V A
 1140 AACAGCTCTGCTGTAAGCTCGATGATACCACTAAGTGGGATTCGCCAAGCGCTCACC
 236 N R V C C K L D D Y H N W D F A K R V T
 1200 GTGACGAGAACGCTCCAGTGCGCAGAACCCAGTCTTGTGTCCTTCGACATCCCG
 256 V T R T T V Q C A E P Q F L F A F D I P
 1260 CGTACATGTTTACATAGCCCGGTGGAAGCGCTGGAATCGGTCTTGATGCTGTAC
 276 P Y M F Y I A A V E K P L E S V L M L Y
 1320 GACGTCGACACATGGAATACGCTTTACCTCCAGTGGCGTATGATACAAACATCTAAA
 296 D V D T W N Y A F T S S G V C I Q T S K
 1380 AAGACGGGTGAGGCTCCCGCGGTGAGTACCAAGTATCTTCGAGAGAGAGTGA
 316 K T G V G L P P G E Y Q C I F E R E S G
 1440 TCGTTATACGGCGGTGCGTGTGAATCGACGCGACATACGATCGAGGAGGAGACCA
 336 S L Y G R P L M V H G S I T I E A K T T
 1500 CACGACTTCGTGAGTAGAGTGGATGCAAAACGTTACCTTCGACGCGGTGTCGAC
 356 H D F V S R V E I Q I T C T F D A V S D
 1560 GCGCGTTCACGCTGGAGCGGTGGAAGACGAGACGCGGTGATCAGAGCGGTTCGCG
 376 G A F T L E R V E D G Q P V Y Q S R V G
 1620 ACTAACGATGCTCTGCTCCGACGACTCCATCTCGATCAACGGTCCGGGTGTCGCGAG
 396 T N E S F V S D D S I S I N G S R L S E
 1680 GGCAAGATCTCGGTTCGCTGATGAGAGAGAGTGGCGTATCGCGCGGTTCGAG
 416 G K I S V S V Y L R E D D G V I G R F R
 1740 TGCCGCGTCCGCTGCGGATGCTTGAATCTGGTATCCGAGCGATCAGCGGTGACAGACCC
 436 C R V R S G C L N L V S R A I T V T K P
 1800 ACCAGGATGGAATACGCTTCGTGACAGAGGAGCGGTACGTCCTTCAGACCAACC
 456 T R M E Y A L V T E R P V T S P O T T T
 1860 ACTACTACTACGACGACCAACACCATCACCCTCCACCGCAAGAACTAGAAAG
 476 T T T T T T T T P S P S P A K K L E E
 1920 ATCAGAATAAAGTCGACGGAACCGACGATGACTTCTGTGGAGACGCAAGAACC
 496 I R I K V D G T D D D F L L E T Q E P
 1980 CTCAAAAGCTACCTACTATCTTATTCGCTCGCGTACGCGGGAAGCGGTCTGGCG
 516 L K S Y P Y Y P L F A R V S A E G R L A
 2040 GCGCTCGCTGTCTTCTAGCCGCGATCTCGTGTGGTCCACATCTCGTTCTGGATC
 536 A V A C F F L A A I L V L H I S F W I
 2100 TCGTCCGCGCGGTGTAACCTGCGGGTTCGCTACGTCACACGTAACGATCATCGACT
 556 S S P P C N L P G C V R H T
 2160 ACTAACACTTACTAATGACTGACCCCGACGATATATAAATAAGCTCTCAAACTAGACA
 2220 cgggtccctcattactctgttatttcgggtcccgacgggtcagggctctcttgccctc
 2280 tctccatgtggcgatactggatcggggcgccatcgggcgatagtcggggcggtcaccac
 2340 cgacggcactgatagccccgggggggtgggattc

FIG. 4. Nucleotide and predicted amino acid sequences of the MCMV FcR gene. The nucleotide sequence of the 2.4-kbp *Bam*HI-*Bam*HI fragment is shown. The predicted mRNA is in capital letters. The TATA box and polyadenylation consensus sequence are in boldface. The predicted amino acid sequence is displayed in the one-letter code. The putative signal sequence and transmembrane region are in boldface. The RGD motif and PEST region are shown by underlined boldface letters. Potential N-linked glycosylation sites are underlined.

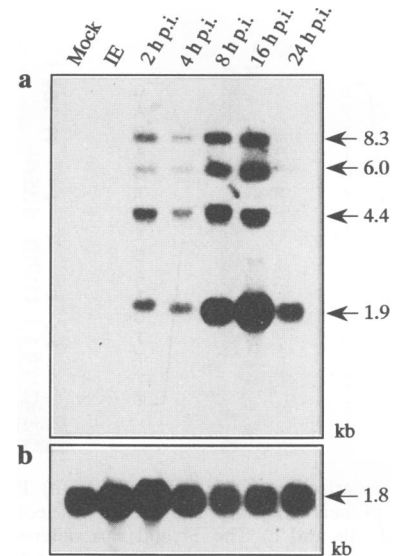


FIG. 5. Transcriptional kinetics of FcR gene expression. (a) Total cellular RNA isolated at different times after infection was hybridized with a strand-specific probe. (b) As a control, filters were rehybridized with a probe specific for a cellular gene, the β -actin gene.

putative transmembrane glycoprotein has 10 potential N-linked glycosylation sites.

At positions 68 to 71, the sequence contains the tripeptide Arg-Gly-Asp (RGD). This sequence represents a ligand recognition motif for several integrins (39, 56). Proximal to the transmembrane region, a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T), a PEST region (43, 45), was identified (positions 458 to 490). This type of region is characteristic for eucaryotic proteins with intracellular half-lives of less than 2 h (44, 46).

Characteristics of the *fcrl* gene. To determine the expression kinetics of the *fcrl* mRNA, total cellular RNA was isolated at different times after infection and Northern (RNA) hybridization was performed. Hybridization with the 32 P-labeled, single-strand-specific probes containing the *fcrl* ORF and generated by runoff synthesis (49) revealed the appearance of four transcripts that were 1.9, 3.3, 4.4, and 6.0 kb (Fig. 5). All four transcripts originate from the same strand, since no hybridization was detected with a probe specific for the opposite strand (data not shown). On the basis of the size of *fcrl* mRNA, which can be predicted from the nucleotide sequence, and on the basis of the gene injection experiments, the 1.9-kb transcript must represent the mRNA for the *fcrl* gene.

Transcription was found to start as early as 2 h after infection and to continue throughout the MCMV replication cycle (Fig. 5a). Maximal transcriptional activity was observed between 8 and 16 h p.i. The same filters were rehybridized with a probe specific for a cellular gene, the β -actin gene, to assess the amount of RNA loaded on the gel (Fig. 5b).

The 5' end of the *fcrl* mRNA was determined by primer extension. A primer was hybridized 150 nt upstream of the putative start site. The resulting cDNA (Fig. 6a) is seen adjacent to the sequencing products with the same primer. The first nucleotide of the mRNA is the T at position 379, 31 nt downstream of the TATA box and 92 nt upstream of the ATG (Fig. 4).

The 3' end of the *fcrl* transcript was determined by reverse

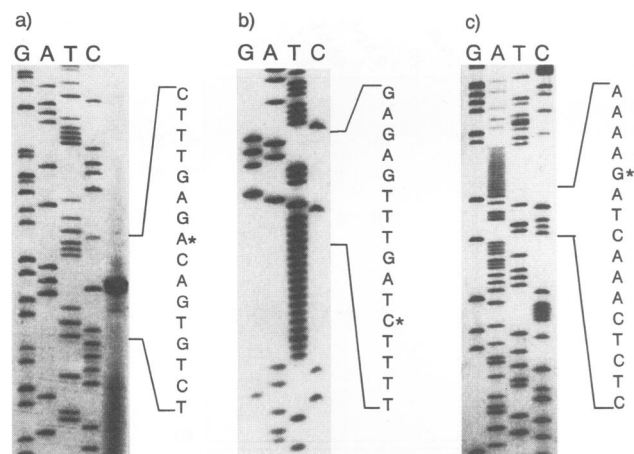


FIG. 6. Characterization of the FcR mRNA. (a) The 5' end was determined by primer extension. The leading nucleotide is marked with an asterisk. (b and c) The 3' end was determined by PCR amplification after reverse transcription. The nucleotide sequences of both strands of the cloned PCR product are shown. The leading nucleotides are marked with asterisks.

transcription of the mRNA and amplification of the cDNA (20) (Fig. 6b and c). In brief, the primer (dT)17-R1-R0 was annealed to RNA and reverse transcription was performed. Amplification of the *fcrl* cDNA was achieved with *fcrl*-specific primer 5'-AGACAGACCATGCCATCG-3' and primer R0 (Fig. 6b and c). After sequencing of this amplified fragment, the 3' end of the *fcrl* mRNA could be located at position 2216, 18 nt downstream of the polyadenylation signal. This predicts a calculated size of 1,838 nt for the *fcrl* mRNA, which is in good agreement with the size of the mRNA identified by Northern hybridization.

Expression of the MCMV FcR by a recombinant vaccinia virus. A recombinant vaccinia virus expressing the MCMV FcR ORF was constructed in order to test whether the cloned gene indeed encodes a protein which binds the Fc fragment of IgG. The FcR gene product synthesized in vaccinia virus FcR-infected cells was characterized by immunoprecipitation of [³⁵S]methionine-labeled proteins with purified IgG and with polyclonal anti-MCMV serum. Proteins with apparent molecular masses of 86 and 88 kDa were equally expressed in cells infected with the recombinant vaccinia virus. They were precipitated by both IgG and MCMV-specific antiserum (Fig. 7). This result proved that the *fcrl* gene identified by microinjection is identical to the gene coding for the protein which is precipitated by IgG from MCMV-infected cell lysates.

Genomic localization of the *fcrl* gene. The *fcrl* gene was located by Southern analysis in the *Hind*III J fragment of the MCMV genome (data not shown) between map units 0.838 and 0.846 (Fig. 8). The gene is located upstream of the *sgg1* gene (33) and a $\beta_{1,3}$ transcript (7) reported by Cardin et al. Four transcripts of significantly different sizes were identified after hybridization with a gene-specific probe (Fig. 5), indicating a complex transcription pattern at this genomic region. All four transcripts coterminate, suggesting the existence of adjacent ORFs upstream of the *fcrl* gene within the *Hind*III I cleavage fragment.

DISCUSSION

This report describes the localization, sequencing, and vector-mediated expression of a gene of MCMV that codes for a

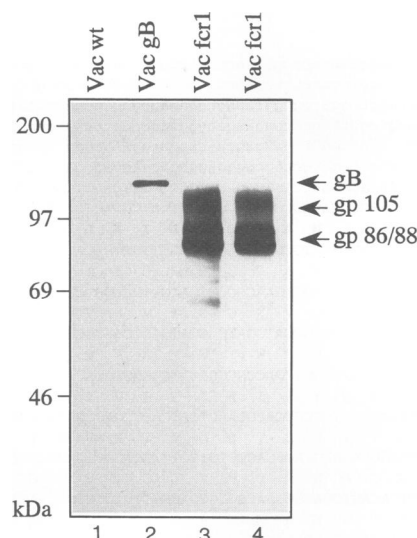


FIG. 7. Expression of the FcR gene by vaccinia virus vector. The cells were infected with wild-type vaccinia virus strain Copenhagen (Vac wt) (lane 1), vaccinia virus gB (Vac gB) (42) (lane 2), and recombinant vaccinia virus expressing the *fcrl* ORF (Vac *fcrl*) (lanes 3 and 4). [³⁵S]methionine-labeled cell lysates (60-min pulse) were precipitated with mouse anti-MCMV serum (lanes 1 to 3) or with mouse IgG (lane 4).

protein with IgG Fc binding properties. In absence of a specific antiserum, the transient expression of this Fc binding phenotype was used to identify the gene. The microinjection technique proved to be convenient and straightforward, because the use of a small amount of genomic viral DNA already digested by restriction enzymes allowed a rather precise location of the gene prior to the cloning procedure. No false-positive results were obtained, probably because the phenotype of injected cells could be directly compared with that of adjacent noninjected cells. The products of the gene termed *fcrl* are early glycoproteins which represent, as judged by sequence, size, and function, bona fide homologs of the gE proteins of HSV-1 and VZV.

No homologous gene of human CMV is known. The strong cytoplasmic binding of human IgG induced by human CMV was reported long ago (21, 27). At the cell surface, the reactivity is stronger with aggregated IgG than with monomeric IgG (19). Generally, the level of surface FcR expression varies among herpesviruses, which led to controversial data with regard to surface expression of VZV FcR (for a review, see reference 30). The surface expression of human CMV FcR is thought to facilitate the entrance of viruses into CMV-infected cells (34). We also observed that for MCMV-infected cells, the intracellular binding of monomeric IgG is much stronger and more consistent than the interaction at the cell membrane (data not shown). In the absence of a specific antiserum for the protein, the strong cytoplasmic expression of the Fc binding property was preferred for identifying the coding region in the genome.

Transcriptional kinetics identified the coding gene, which was termed *fcrl*, as an early gene that is expressed from 2 h p.i. on throughout the replication cycle. The determined length of 1,838 nt for the *fcrl* mRNA is in good agreement with the length of 1.9 kb for the polyadenylated mRNA found early after infection; the polyadenylated mRNA is the shortest member of a family of four early transcripts. This RNA family

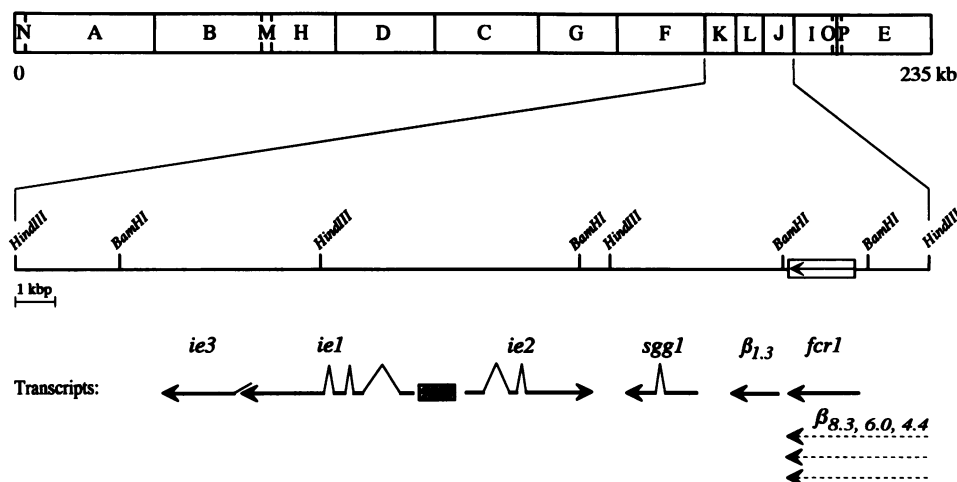


FIG. 8. Genomic localization of the MCMV *fcr1* gene. Shown is the *Hind*III cleavage map of the MCMV genome (top) and the known transcripts encoded by the *Hind*III K, L, and J fragments (bottom). Localization of the *fcr1* ORF is indicated by an open box with an arrow. The other early (β) transcripts are represented by dashed lines and were not further characterized.

is transcribed from the same strand in the same direction, and all four transcripts probably cotermminate with the *fcr1* mRNA. This conclusion was based on the finding that after cDNA synthesis and PCR amplification, only a single fragment that defined the 3' end of the *fcr1* mRNA was obtained. Although this PCR method amplifies fragments in a certain size range, it is unlikely that a member of this mRNA family terminates further downstream, because another 1.3-kb early RNA has been mapped to the adjacent region (7). Multiple and longer transcripts from the same strand also occur in the regions of the gB and polymerase genes of MCMV and human CMV (17, 28, 31, 42, 47) as well as in the region encoding the gH of MCMV (41).

The *fcr1* gene predicts an ORF with the potential to encode a protein of 569 amino acids. This protein is a type I glycoprotein with an N-terminal secretory signal sequence of 17 residues and a transmembranal region extending from positions 535 to 552 followed by a cytoplasmic tail of 17 amino acids. The size of an early glycoprotein which was detected in infected cells and which migrates at a relative mobility of 64 kDa after deglycosylation is also in good agreement with the predicted molecular mass of the *fcr1* gene product, and proof for identity was provided by vaccinia virus expression of *fcr1*. Because of the lack of a specific antibody, the characterization of the *fcr1* gene product has been limited to precipitation by IgG. Work to define the conditions of IgG binding by the *fcr1* product and its biochemical properties in more detail is in progress. Studies of the biochemical properties of human CMV proteins with Fc binding functions revealed four proteins of 38, 50, 65, and 130 kDa (5). The latter two proteins were also found in virions. Stannard and Hardie (48) detected Fc-binding polypeptides of 33 and 69 kDa and also associated Fc binding activity with the tegument of human CMV. Our studies of the proteins expressed after infection have been restricted to short-term pulse-chase experiments. Analysis of proteins expressed by vaccinia virus also showed gp86/88 and gp105. This does not exclude further modifications, since we have observed that MCMV glycoproteins can be processed differently after expression by MCMV and by vaccinia virus (42).

If the FcR of CMV is homologous with the receptor of alphaherpesviruses, which is a heterodimer composed of the

glycoproteins gE and gI (25), then the protein we describe is probably the homolog of gE. This conclusion is based on the facts that only the gE protein of HSV-1 has intrinsic Fc binding activity and that its interaction with gI endows gE with the ability to interact with both IgG monomers and aggregates. At 550 residues for HSV-1 gE and 623 for VZV gE, all three proteins fall into a similar size class. The two genes for HSV-1 and VZV gE have an identity of 19% and a similarity of 34%. A comparison of the amino acid sequence of HSV-1 gE with the sequence of *fcr1* reveals an identity of 14.3% and a similarity of 38.6%, and a comparison of the VZV gE sequence with the *fcr1* sequence reveals an identity and a similarity of 13.9 and 41.1%, respectively (data not shown). In addition, the N-terminal region of *fcr1* contains the RGD tripeptide cell adhesion motif shared by many cell adhesion molecules (13) and which is absent from the Fc γ R of HSV-1 and VZV. Finally, the sequence contains a PEST motif which is typical for proteins with a short half-life (43, 45). The functional relevance of these sequence motifs and of the Fc binding property is unknown at present. At least three possibilities exist. The first is that the FcR indeed has a function that allows the virus to evade immune control, a function similar to the effect of the so-far-unknown early gene products affecting the export of peptide-loaded major histocompatibility complex class I molecules (9). Second, the FcR property could be irrelevant for the in vivo function of the gene product and could perhaps indicate a propensity to interact with certain glycoproteins. The finding that the surface expression of the VZV FcR is rather weak and that in CMV the protein is mainly in the cytoplasm and is associated with the tegument of the virion may indicate such a possibility. The third possibility is that the protein has more than one function. Recent work by Dubin et al. (14) suggesting a homology of a domain of the HSV-1 gE that is involved in Fc binding with mammalian FcR sequences seems to support the immunological role.

Our interest in the potential immunoevasive role of herpesvirus FcR was raised when we found that antibodies are not essential to combat acute MCMV infection and to establish viral latency (26). This could indicate either that the antibody-binding FcR function is irrelevant during MCMV infection or that this property indeed makes an important contribution and is directly responsible for the only apparent irrelevance of

antibodies during acute infection. Under the assumption that the homologous genes should be nonessential for the virus, we have started to construct MCMV mutants by insertional mutagenesis. Although further testing is required, it is already clear that a mutant harboring a truncated form of the *fer1* gene still replicates in tissue culture (49a). Thus, the phenotype of a CMV infection in the presence and absence of FcR functions will soon become testable in a natural virus host model.

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